

Blood Coagulation, Fibrinolysis and Cellular Haemostasis

Comprehensive analysis of ADAMTS13 in patients with liver cirrhosis

Masahito Uemura^{1*}, Yoshihiro Fujimura^{2*}, Masanori Matsumoto², Hiromichi Ishizashi², Seiji Kato², Tomomi Matsuyama¹, Ayami Isonishi², Masatoshi Ishikawa¹, Masato Yagita⁴, Chie Morioka¹, Hitoshi Yoshiji¹, Tatsuhiro Tsujimoto¹, Norio Kurumatani³, Hiroshi Fukui¹

¹Third Department of Internal Medicine, ²Department of Blood Transfusion Medicine, ³Department of Community Health and Epidemiology, Nara Medical University, Kashihara, Nara, Japan; ⁴Kitano Hospital, Osaka, Japan

Summary

Decreased plasma ADAMTS13 activity (ADAMTS13:AC) results in the accumulation of unusually large von Willebrand factor multimer (UL-VWFm) and the formation of platelet thrombi. It remains controversial whether or not plasma ADAMTS13:AC decreases in patients with liver cirrhosis (LC), and its relationship to clinical features has not been fully investigated. We measured ADAMTS13:AC and its related parameters in plasma in 33 patients with chronic hepatitis (CH) and in 109 patients with LC. ADAMTS13:AC decreased with increasing severity of liver disease (controls means 100%, CH 87%, Child A-LC 79%, Child B-LC 63%, and Child C-LC 31%), and showed severe deficiency (<3% of controls) in five end-stage LC. Activities measured by act-ELISA strongly correlated with those determined by the VWFm assay and ADAMTS13 antigen. Multivariate analysis showed Child-Pugh score and spleen volume inde-

pendent factors contributing to ADAMTS13:AC. VWFm patterns were normal in 53% of cases, degraded in 31%, and unusually large in 16%. Patients with unusually large VWFm had the lowest ADAMTS13:AC as well as the highest Child-Pugh score, serum creatinine and blood ammonia levels. Plasma inhibitor against ADAMTS13 detected in 83% of patients with severe to moderate ADAMTS13:AC deficiency mostly showed marginal zone between 0.5 and 1.0 BU/ml. The IgG-type autoantibodies specific to plasma derived-ADAMTS13 was detected by Western blot in only five end-stage LC with severe ADAMTS13:AC deficiency. In conclusion, both plasma ADAMTS13 activity and antigen levels decreased with increasing severity of cirrhosis. An imbalance between the decreased ADAMTS13:AC and its increased substrate may reflect the predisposing state for platelet thrombi formation in patients with advanced LC.

Keywords

ADAMTS13 activity, liver cirrhosis, von Willebrand factor, thrombocytopenia, inhibitor

Thromb Haemost 2008; 99: 1019–1029

Introduction

ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) is a metalloproteinase that specifically cleaves the Tyr1605-Met1606 bond of von Willebrand factor (VWF) A2 domain (1, 2). VWF is exclusively synthesized in vascular endothelial cells and is secreted into the circulation as unusually large VWF multimers (UL-VWFms), which are most biologically active to aggregate platelets and form platelet thrombi under high shear stress (3). ADAMTS13 rapidly cleaves UL-VWFms into smaller VWF multimers,

which are less biologically active, and thereby prevents platelet hyperaggregation and thrombi formation (3). Congenital deficiency of ADAMTS13 activity (ADAMTS13:AC) caused by its gene mutations (1, 4), and acquired deficiency due to the development of the neutralizing autoantibodies, ADAMTS13 inhibitors (ADAMTS13:INH) (5, 6), result in thrombotic thrombocytopenic purpura (TTP), a life-threatening generalized disease (7). The *ADAMTS13* gene was originally cloned using liver cell libraries (2), and it was later shown that the enzyme is produced exclusively in hepatic stellate cells (HSCs) (8).

Correspondence to:
Masahito Uemura, MD
Third Department of Internal Medicine
Nara Medical University, Kashihara
Nara, 634-8522, Japan
Tel.: +81 744 22 3051 ext. 3415, Fax: +81 744 24 7122
E-mail: muemura@naramed-u.ac.jp

*These authors contributed equally to this study.

Financial support:
This work was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (MU, YF) and from the Ministry of Health and Welfare of Japan for Blood Coagulation Abnormalities (YF).

Received January 5, 2008
Accepted after minor revision April 13, 2008

Republished online May 7, 2008
doi:10.1160/TH08-01-0006

Variable	Chronic hepatitis (n=33)	Liver cirrhosis		
		Child A (n=35)	Child B (n=33)	Child C (n=41)
Age (years)	56.6 ± 12.4	66.4 ± 7.8 ^b	63.6 ± 8.3 ^a	64.9 ± 15.8 ^a
Sex (male/female)	17/16	25/10	17/16	23/18
Cause of liver disease				
HCV/HBV/Alcohol/PBC/Cryptogenic	29/3/0/0/1	24/4/4/0/3	20/7/2/0/4	23/5/5/4/4
Child-Pugh score	—	5.5 ± 0.5	7.9 ± 1.0 ^c	11.6 ± 1.5 ^e
Spleen volume (mm ³)	219 ± 123	323 ± 181 ^a	399 ± 250 ^b	559 ± 248 ^{b, c, d}
Ascites (-/easily mobilized/refractory)	0	35/0/0	21/10/2	8/6/27
Hepatorenal syndrome (+)	0	0	0	10
Encephalopathy (+)	0	0	9	33
Esophageal varices (-/mild/severe) ^f	0	10/12/13	3/7/23	3/6/32
Hepatocellular carcinoma (+)	2	22	16	19
JIS score ^g	0, 0	1.4 ± 0.9	2.8 ± 1.0 ^c	3.7 ± 1.1 ^{c, d}

The data are expressed as mean ± SD. HCV, hepatitis C virus; HBV, hepatitis B virus; PBC, primary biliary cirrhosis.

^ap<0.01 and ^bp<0.001 vs. patients with chronic hepatitis, respectively. ^cp<0.001 vs. cirrhotics with Child A. ^dp<0.01 and ^ep<0.001 vs. cirrhotics with Child B, respectively. ^fMild or severe esophageal varices indicate lesions without or with endoscopic signs of impending variceal rupture, respectively. ^gThe Japan Integrated Staging score obtained via the summation of Child-Pugh score and tumor stage score (32).

Table 1: Clinical data of patients with chronic liver diseases.

Alternatively, increased plasma levels of VWF antigen (VWF:Ag)(9, 10), and thrombocytopenia are commonly seen in patients with advanced LC (11–13). Moreover, cases of advanced LC are often complicated by thrombosis in one or more organs in addition to the portal and hepatic veins (14–16), suggesting a predisposition toward thrombogenesis in advanced LC.

Regarding the possibility of a relationship of ADAMTS13 and various liver diseases, Mannucci et al. (17) originally reported a significant reduction of the ADAMTS13:AC in advanced LC. Subsequently, we have shown that this activity is significantly reduced in patients with hepatic veno-occlusive disease (18), alcoholic hepatitis (19), and those undergoing living-donor related liver transplantation (20). Furthermore, HCV-related cirrhosis patients typically develop TTP with ADAMTS13:INH (21). In cases of advanced LC, however, the recent two studies reported apparently opposite results on plasma levels of ADAMTS13:AC: in one study (22), activity was unchanged, whereas in the other it was reduced (17, 23). The authors of the former study further reported that plasma levels of VWF:Ag were increased, but the higher molecular weight multimer was more degraded than normal controls, thus maintaining normal levels of enzyme-to-substrate (ADAMTS13/UL-VWFMs) ratio to maintain blood fluidity. The study employed the collagen binding assay for ADAMTS13:AC, and the assay has been established, but it is possible that compounds in the tested samples affected the assays in a manner that is not well-understood. In the classic VWF assay, a gold standard method for ADAMTS13:AC (24), a high concentration of free hemoglobin is well-known to inhibit the enzyme activity (25); likewise, in fluorogenic FRETs-VWF73 assays (26), both bilirubin and chylomicron in tested samples block the activity (27). More recently, however, a chromogenic enzyme-linked immunoassay for ADAMTS13:AC (ADAMTS13-act-ELISA) was shown to be totally insensitive to the presence of such compounds (28).

We have performed comprehensive studies in a large population of patients with chronic liver diseases (n=142), paying particular attention to ADAMTS13 and VWF; plasma levels of ADAMTS13:AC, ADAMTS13 antigen (ADAMTS13:AG), ADAMTS13:INH, VWF:Ag, VWF ristocetin cofactor activity (VWF:RCo), and analysis of plasma UL-VWFMs, in order to explore the relationship between ADAMTS13:AC, the clinical features, and laboratory findings of patients with liver cirrhosis. Furthermore, we examined the presence or absence of immunoglobulin G (IgG)-type autoantibodies specific to plasma derived-ADAMTS13 by Western blot in patients with ADAMTS13:INH in plasma.

Materials and methods

Patients

A total of 142 patients with chronic liver diseases were included in this study, of whom 33 had biopsy-proven chronic hepatitis and 109 had LC, including a case with TTP (21) (Table 1). Patients with a known history of coagulopathies, sepsis, or platelet disorders were excluded. The origin of liver disease was hepatitis C virus (HCV) in 96 cases; hepatitis B virus (HBV) in 19; alcohol abuse in 11; primary biliary cirrhosis (PBC) in four; and cryptogenic in 12. The diagnosis of cirrhosis was based on physical findings, laboratory tests, and in many cases had been confirmed by histological criteria. Of the LC patients, 35 were Child A, 33 were Child B, and 41 were Child C, according to Child-Pugh's criteria (29). Spleen volume, determined by computed tomography scans (30), increased as liver disease progressed. Ascites was easily mobilized in 16 patients and refractory in 29, 10 of whom finally progressed to hepatorenal syndrome according to the criteria described previously (31). Spontaneous bacterial peritonitis (SBP) occurred in 10 patients with refractory ascites, and in seven patients this was complicated by hepatorenal syn-

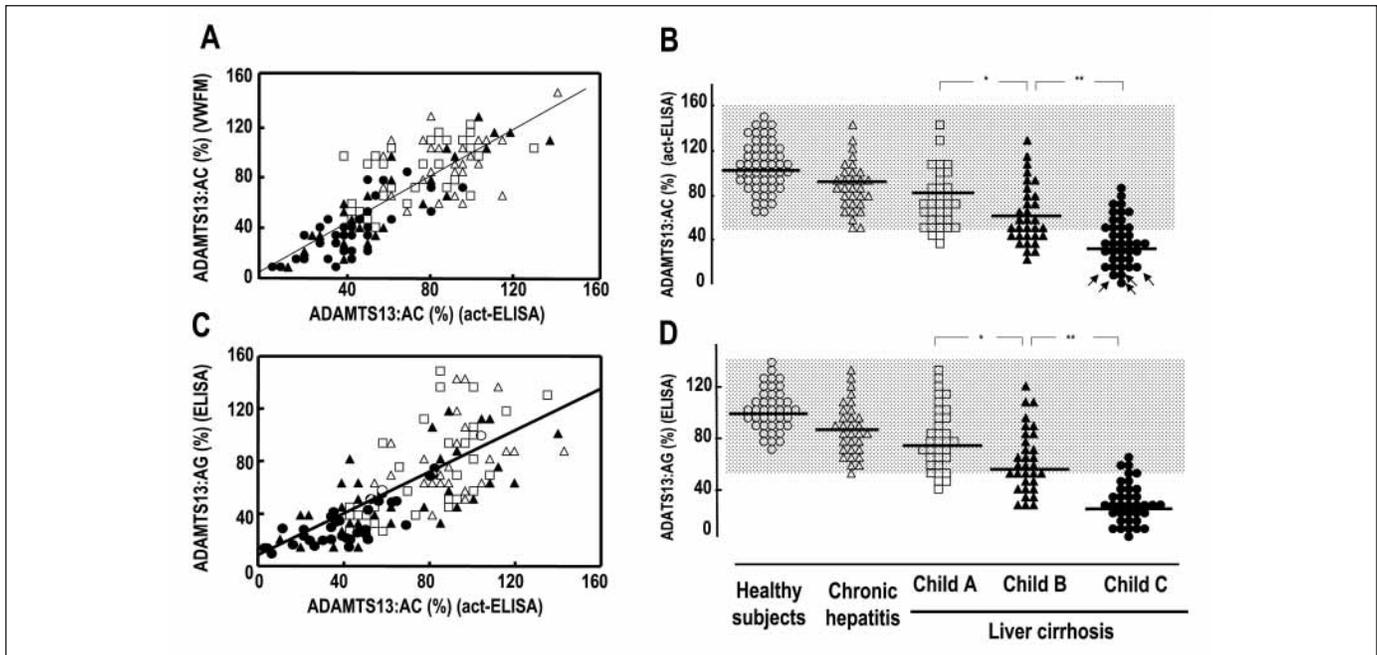


Figure 1: Plasma levels of ADAMTS13:AC and ADAMTS13:AG in patients with chronic liver diseases. The plasma levels of ADAMTS13:AC determined by two methods, the VWFM assay and ADAMTS13-act-ELISA, were in good accordance ($r=0.841$, $p<0.001$), although some discrepant results were observed (A). Severe deficiency in ADAMTS13:AC ($<3\%$) was seen in five LC patients with Child C by the VWFM assay, but by the act-ELISA they ranged from <0.5 to 15.9% of the normal control (B, shown by arrows). Plasma ADAMTS13:AC determined by the ELISA progressively decreased with worsening cirrhosis (B). In addition, the plasma levels of ADAMTS13:AG quantified by the

ELISA highly correlated with ADAMTS13:AC measured by the act-ELISA ($r=0.715$, $p<0.001$) (C). The ADAMTS13:AG levels also decreased with increasing cirrhosis severity (D). Open circles, normal controls; open triangles, chronic hepatitis; open squares, cirrhosis with Child A; closed triangles, cirrhosis with Child B; closed circles, cirrhosis with Child C. Shaded area shows normal range. ADAMTS13:AC=ADAMTS13 activity, ADAMTS13:AG=ADAMTS13 antigen, VWFM=von Willebrand factor multimer. * $p<0.05$, and ** $p<0.001$ significantly different between the two groups.

drome. Hepatic encephalopathy grade II or greater developed in 42 patients. Sixty-eight LC patients had endoscopic signs of impending variceal rupture. Hepatocellular carcinoma (HCC) was found in two patients with chronic hepatitis and 57 cirrhosis patients. HCC was graded by the Japan Integrated Staging (JIS) score (32) obtained via the summation of tumor stage score and Child-Pugh score (Table 1). All subjects gave informed consent to participate in the study. The study protocol was approved by the Nara Medical University Hospital Ethics Committee.

Determination of ADAMTS13:AC, ADAMTS13:AG, VWF:Ag, VWF:RCo, UL-VWFMs and ADAMTS13:INH

Blood was obtained from the patients at the time of admission or during a hospital stay. Samples were stored in plastic tubes containing $1/10^{\text{th}}$ volume of 3.8% sodium citrate. Platelet-poor plasma prepared by centrifugation at $3000 \times g$ at 4°C for 15 minutes was stored in aliquots at -80°C until analysis. Plasma ADAMTS13:AC was determined by both a classic VWFM assay (24, 33) and a sensitive chromogenic ELISA (ADAMTS13-act-ELISA: Kainos Inc., Tokyo, Japan) (28). The normal value was $102 \pm 23\%$ in the VWFM assay (33) and $99 \pm 22\%$ in the act-ELISA (28). ADAMTS13:AG was measured by a sandwich ELISA using two murine monoclonal antibodies (mAbs) as previously reported (34), and its normal value was $106 \pm 39\%$. In five LC patients whose ADAMTS13:AC was less than 3% in the

VWFM assay, Western blot (WB) analysis was performed to quantify ADAMTS13:AG using the murine anti-ADAMTS13 mAb WH2-11-1 (IgG1- κ) (35). Plasma VWF:Ag was measured by a rabbit polyclonal sandwich ELISA (Dako, Glostrup, Denmark), and its normal level was $100 \pm 53\%$ ($n=60$, 20–39 years of age). VWF:RCo was determined as described (36), and its normal value was $100 \pm 15\%$. In 49 LC patients with lower ADAMTS13:AC (less than 50% of normal control), plasma UL-VWFMs were analyzed by a vertical SDS- 1.0% agarose gel electrophoresis system (37), and evaluated using NIH image J. ADAMTS13:INH was evaluated using heat-inactivated plasma at 56°C for 30 minutes (5, 6). One Bethesda unit of inhibitor was defined as the amount of plasma that reduces ADAMTS13:AC to 50% of the control (38), and its titer was defined to be significant at >0.5 Bethesda Units (BU)/ml.

Detection of IgG-type autoantibodies specific to plasma derived-ADAMTS13 by WB

In order to differentiate between IgG associated with autoantibodies against ADAMTS13 and high IgG concentration, we tried to detect IgG-type autoantibodies specific to plasma derived-ADAMTS13 by WB. Plasma-derived (pd)-ADAMTS13 was purified using A10-agarose immunoaffinity chromatography followed by size-exclusion chromatography (39). The A10 was the murine anti-ADAMTS13 monoclonal antibody, which rec-

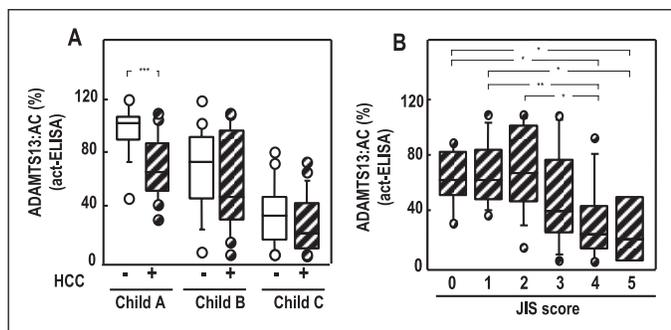


Figure 2: Plasma levels of ADAMTS13:AC with and without hepatocellular carcinoma. Child A LC patients with HCC had reduced ADAMTS13:AC compared to those without HCC, but there were no differences between Child B and C LC patients with and without HCC (A). ADAMTS13:AC was lower in patients with JIS score 4 and score 5 compared to patients with score 0, score 1, and score 2 (B). Open box, cirrhosis patients without HCC; shaded box, with HCC. ADAMTS13:AC=ADAMTS13 activity, HCC= hepatocellular carcinoma, JIS=The Japan Integrated Staging score obtained via the summation of Child-Pugh score and tumor stage score (32). *p<0.05, **p<0.01, and ***p<0.001 significantly different between the two groups.

ognizes an epitope in the disintegrin domain, totally inhibiting enzyme activity at a final concentration of 50 µg/ml (8). Purified pd-ADAMTS13 had a specific activity of 302 units/mg. SDS-5% polyacrylamide gel electrophoresis (PAGE) analysis

revealed a 170 kD-band before and a 190 kD-band after reduction. During storage at -80°C, however, the oxidized 170 kD-band occasionally degraded into fragments with smaller molecular masses of 130~150 kDa. To detect IgG-type autoantibodies specific for purified pd-ADAMTS13, 0.15 µg pd-ADAMTS13 per lane was separated by SDS-5%PAGE under non-reducing conditions, then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking nonspecific binding with 5% skim milk, PVDF membranes were cut longitudinally into small pieces (3 x 800 mm). Each strip was incubated overnight at 4°C with 3 ml 5% skim milk containing 50 µl heat-treated plasma obtained from each patient who had ADAMTS13:INH more than 0.5 Bethesda Units (BU)/ml. The heat-treated plasma was prepared by incubation at 56°C for 30 min. After centrifugation, the supernatant was used in assays. Human IgG bound to the purified pd-ADAMTS13 on PDVF membranes was detected using a horse-radish peroxidase (HRP)-conjugated anti-human IgG antibody (ICN Pharmaceuticals Inc., Aurora, OH, USA). Binding was visualized by chemiluminescence with Western Lightning Chemiluminescence reagent (Perkin-Elmer Life Science Inc., Boston, MA, USA) and imaged by X-ray autoradiography (Eastman Kodak, Rochester, NY, USA). Heated plasma from a patient with acquired idiopathic TTP with IgG inhibitors against ADAMTS13 was used as a positive control, while plasma from a normal individual without ADAMTS13:INH was used as a negative control.

Table 2: Correlation coefficients between plasma ADAMTS13:AC and clinical variables in patients with chronic liver diseases.

Variables	Correlation coefficients	P-value
Child-class	-0.597	<0.0001
Child-Pugh score	-0.593	<0.0001
Hemoglobin (g/dl)	0.490	<0.0001
Spleen volume (cm ³)	-0.470	<0.0001
Prothrombin time (%)	0.451	<0.0001
Serum albumin (g/dl)	0.418	<0.0001
Platelet count (x10 ⁴ /mm ³)	0.413	<0.0001
C-reactive protein (mg/dl)	-0.395	<0.0001
Blood ammonia (µg/dl)	-0.382	0.0005
Total cholesterol (mg/dl)	0.361	<0.0001
Blood urea nitrogen (mg/dl)	-0.353	<0.0001
Serum indirect bilirubin (mg/dl)	-0.329	<0.0001
Serum creatinine (mg/dl)	-0.326	0.0001
Interleukin-6 (pg/ml)	-0.267	0.002
Serum total bilirubin (mg/dl)	-0.256	<0.005
Lactate dehydrogenase (IU/l)	-0.240	<0.005
White blood cell count (/mm ³)	-0.237	<0.005

ADAMTS13:AC, ADAMTS13 activity.

Measurements of cytokines

Plasma concentrations of cytokines, tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and interleukin 8 (IL-8), were determined with commercially available kits (Immunoassay Kits, BioSource International, Camarillo, CA, USA).

Statistical analysis

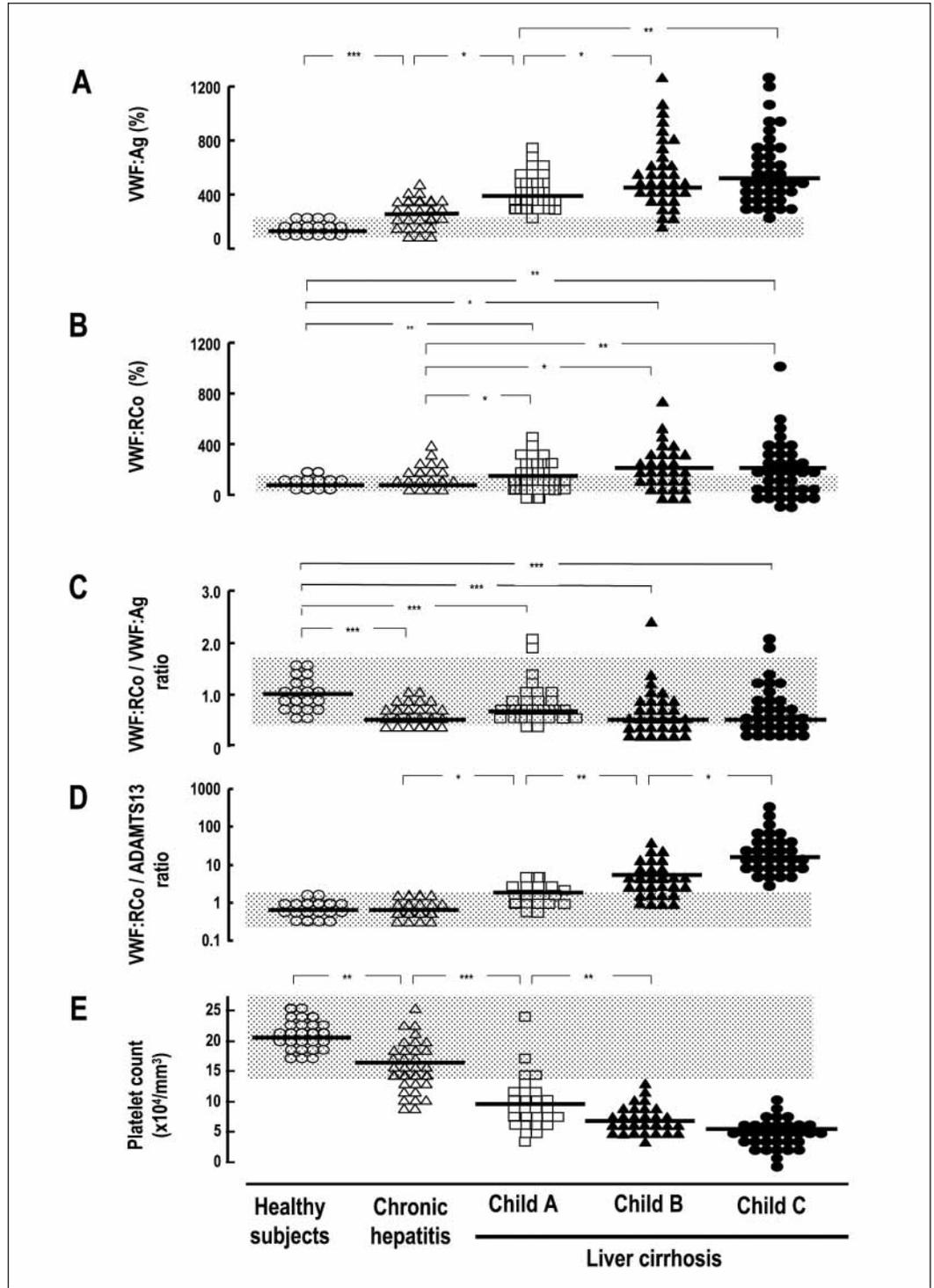
Differences between paired and unpaired groups were analyzed using Student's *t* test. Correlations were calculated by Spearman rank test. To determine which clinical parameters independently correlated with plasma ADAMTS13:AC, we applied a stepwise selection procedure based on multiple regression analysis. The analyses were carried out using Statview statistical software (version 5.0; SAS Institute, Cary, NC, USA). The data are expressed as the mean ± SD. A two-tailed p-value less than 0.05 was considered significant.

Results

Plasma levels of ADAMTS13:AC and ADAMTS13:AG

Plasma levels of ADAMTS13:AC determined by VWFM assay were 56 ± 34% in LC patients, which was significantly lower than those of both healthy subjects (p<0.001) and patients with chronic hepatitis (87 ± 27%, p<0.001). The ADAMTS13:AC levels progressively decreased with worsening cirrhosis; 79 ± 25% in Child A, 63 ± 34% (p<0.05) in Child B, and 31 ± 22% (p<0.001) in Child C. The levels of ADAMTS13:AC determined by the act-ELISA were in good accordance with those measured by the VWFM assay (r=0.841, p<0.001) (Fig. 1A); these values were 91 ± 22% in chronic hepatitis, 80 ± 24% in Child A, 65 ±

Figure 3: Plasma levels of VWF:Ag, VWF:RCo, VWF:RCo/VWF:Ag ratio, and VWF:RCo/ADAMTS13:AC ratio, and platelet count in patients with chronic liver disease. The VWF:Ag increased with the progression of chronic liver diseases, but the difference between Child B and C did not reach statistical significance (A). The VWF:RCo is higher in LC patients than in patients with chronic hepatitis and healthy subjects, but it did not differ among subgroups within LC (B). The VWF:RCo relative to VWF:Ag was lower in patients with chronic hepatitis and LC than in healthy subjects, but no differences were found among subgroups within LC (C). Furthermore, the VWF:RCo relative to ADAMTS13:AC progressively increased with worsening chronic liver disease (D). Platelet count decreased with the severity of chronic liver diseases, but no difference was found between Child B and C. VWF:Ag= von Willebrand factor antigen, VWF:RCo= von Willebrand factor ristocetin cofactor activity, ADAMTS13:AC=ADAMTS13 activity. Shaded area shows normal range. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ significantly different between the two groups.



31% in Child B, and $40 \pm 22\%$ in Child C (Fig. 1B). Some discrepancies were observed: for example, deficiency in ADAMTS13:AC (<3%) by the VWFm assay was seen in five LC patients with Child C, but by the act-ELISA these patients ranged from <0.5 to 15.9% of the normal control (Fig. 1B, shown by arrows). Because the two types of assays were in good agreement, plasma levels of ADAMTS13:AC described in this paper are the values determined by act-ELISA unless otherwise noted.

Furthermore, the plasma levels of ADAMTS13:AG were highly correlated with ADAMTS13:AC ($r=0.715$, $p < 0.001$) (Fig. 1C). The levels of ADAMTS13:AG were significantly lower in LC patients ($52 \pm 33\%$), than in healthy subjects ($p < 0.001$) and patients with chronic hepatitis ($81 \pm 28\%$, $p < 0.001$). These values also decreased with increasing cirrhosis severity (Child A, $74 \pm 36\%$; Child B, $55 \pm 29\%$, $p < 0.05$; Child C, $30 \pm 15\%$, $p < 0.001$) (Fig. 1D).

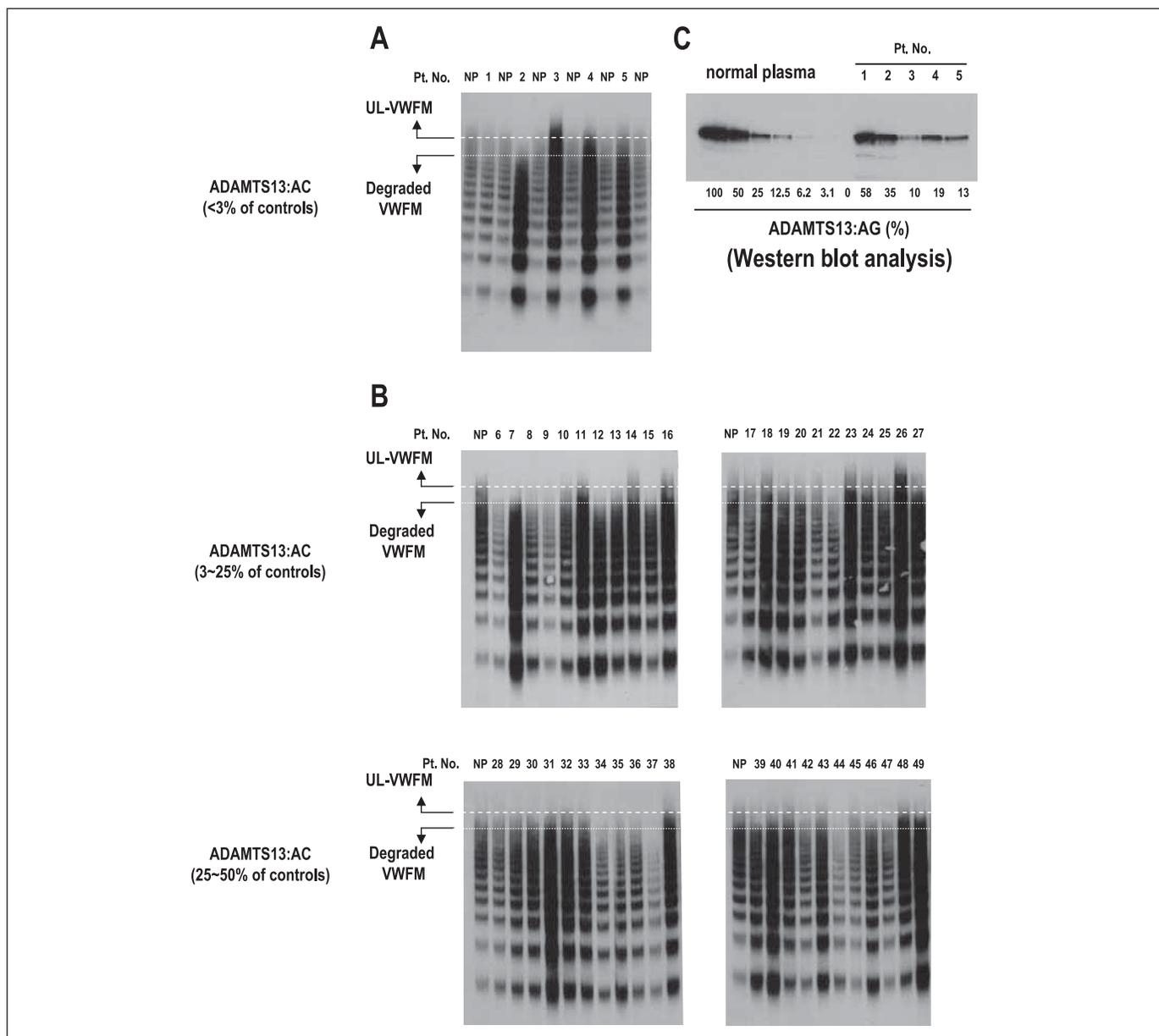


Figure 4: Plasma VWF multimer in 49 LC patients with severe to mild deficiency of ADAMTS13:AC and Western blot analysis of ADAMTS13 in five LC patients with severe deficiency of ADAMTS13:AC. The VWF multimer was analyzed by a vertical SDS-1.0% agarose gel electrophoresis system (A, B). Five patients (patients no. 1–5) (A) were originally identified as a severe deficiency of plasma ADAMTS13:AC by the VWF assay. Twenty-two patients (patients nos. 6–27) showed a moderate deficiency (3–25% of the control), and the remaining 22 patients (nos. 28–49) mild deficiency (25–50% of the control) of plasma ADAMTS13:AC by both methods of VWF assay and the act-ELISA, without discordant results. There were three differ-

ent patterns including degraded-, normal-, and UL-VWFM. Out of these 49 patients, 26 (53.1%) showed normal VWFs, 15 (30.6%) degraded-VWFs, and the remaining eight (patients nos. 3, 4, 11, 14, 16, 18, 23, and 26) (16.3%) UL-VWFs. ADAMTS13:AC = ADAMTS13 activity, VWF= von Willebrand factor multimer, UL-VWFM = unusually large von Willebrand factor multimer, NP= normal control plasma. Panel C showed the Western blot analysis of ADAMTS13:AG, which was performed using a murine anti-ADAMTS13 monoclonal antibody. ADAMTS13:AG was quantified from 10% to 58% in five LC patients according to the intensity of blotting using NIH image J. The left part of panel C shows a standard curve using diluted normal plasma.

Plasma levels of ADAMTS13:AC versus clinical variables

In order to investigate the effects of HCC complicated with LC on plasma levels of ADAMTS13:AC, the patients were divided into two groups, with and without HCC. As shown in Figure 2A, patients of Child A LC patients with HCC had a significantly

lower ADAMTS13:AC than those without HCC ($69 \pm 22\%$ versus $103 \pm 11\%$, $p < 0.001$), but there were no differences between Child B and Child C LC patients with and without HCC. Furthermore, as shown in Figure 2B, plasma ADAMTS13:AC levels were lower in patients with JIS score 4 ($32 \pm 28\%$, $p < 0.05$) and score 5 ($26 \pm 29\%$, $p < 0.05$) than in patients with score 0 ($67 \pm$

Table 3: Comparison of clinical parameters among cirrhotic patients according to VWF multimer patterns.

Variables	VWF multimer pattern			A vs. B	A vs. C	B vs. C
	degraded ^a	normal ^b	unusually large ^c			
	n=15	n=26	n=8			
ADAMTS13:AC (%) (act-ELISA)	47 ± 24	44 ± 13	26 ± 14	n.s.	p<0.05	p<0.01
VWF:RCo (%)	110 ± 92	196 ± 134	216 ± 110	p<0.05	p<0.05	n.s.
Child-Pugh score	8.6 ± 2.5	10.9 ± 2.1	12.4 ± 1.7	p<0.01	p<0.005	n.s.
Serum albumin (g/dl)	3.07 ± 0.54	2.85 ± 0.54	2.59 ± 0.25	n.s.	p<0.05	n.s.
Cholinesterase (IU/l)	126 ± 62	78 ± 64	60 ± 36	p<0.05	p<0.02	n.s.
Total cholesterol (mg/dl)	142 ± 51	93 ± 45	88 ± 40	p<0.01	p<0.03	n.s.
Hemoglobin (g/dl)	11.0 ± 1.7	9.3 ± 2.0	8.9 ± 1.7	p<0.02	p<0.02	n.s.
Serum creatinine (mg/dl)	1.06 ± 0.72	1.11 ± 0.79	2.43 ± 2.16	n.s.	p<0.05	p<0.03
Blood urea nitrogen (mg/dl)	22 ± 17	30 ± 21	74 ± 62	n.s.	p<0.01	p<0.01
Blood ammonia (μg/dl)	87 ± 50	100 ± 39	144 ± 53	n.s.	p<0.05	p<0.05

VWF, von Willebrand factor; ADAMTS13:AC, ADAMTS13 activity; ELISA, enzyme-linked immunosorbent assay; VWF:RCo, VWF ristocetin cofactor activity; n.s., not significant.

24%), score 1 (65 ± 37%), and score 2 (69 ± 22%). In addition, the levels of ADAMTS13:AC were significantly lower in LC patients with the following clinical conditions than those without; hepatic encephalopathy (28 ± 24% versus 71 ± 29%, p<0.001), hepatorenal syndrome (13 ± 12% versus 61 ± 33%, p<0.001), and severe esophageal varices (46 ± 33% versus 75 ± 34%, p<0.05). Moreover, patients with refractory ascites had lower ADAMTS13:AC (25 ± 17%) than those without ascites (70 ± 30%, p<0.001) or those with easily mobilized ascites (56 ± 37%, p<0.001).

We next performed a univariate analysis using all patients with chronic hepatitis and liver cirrhosis, excluding a patient with TTP, and the 17 examined variables significantly correlated with ADAMTS13:AC (Table 2). We further performed a multivariate analysis using all significant baseline parameters determined by the univariate analysis except Child-Pugh score. Spleen volume, blood ammonia, and serum creatinine independently correlated with ADAMTS13:AC (r=0.690, p<0.0001). As a second step, the three parameters that contribute to the Child-Pugh classification (total bilirubin, albumin, and prothrombin time) were replaced by the Child-Pugh score. As a result, the Child-Pugh score and spleen volume were independently selected (r=0.548, p<0.0001).

Plasma levels of VWF:Ag and VWF:RCo, the ratios of VWF:RCo/VWF:Ag and VWF:RCo/ADAMTS13:AC, and platelet count

Plasma levels of VWF:Ag were significantly higher in LC patients (421±249%) than in patients with chronic hepatitis (245 ± 135%, p<0.001) and healthy subjects (p<0.001) (Fig. 3A); in subgroups of LC patients, it was 320 ± 174% in Child A, 436 ± 267% (p<0.05) in Child B, and 486 ± 254% (p<0.01) in Child C. Like VWF:Ag, plasma levels of VWF:RCo were higher in LC patients (205 ± 168%) than in patients with chronic hepatitis (101 ± 86%, p<0.005) and healthy subjects (100 ± 15%, p<0.01), but did not differ among subgroups of LC patients: 186 ± 137% in Child A, 198 ± 172% in Child B, and 227 ± 187% in Child C (Fig. 3B).

The ratio of VWF:RCo to VWF:Ag was lower in patients with chronic hepatitis (0.45 ± 0.27, p<0.001) and LC (0.54 ± 0.45, p<0.001) than in healthy subjects (1.1±0.42), but no differences were found among LC patients with Child A (0.63±0.49), Child B (0.50±0.46) and Child C (0.51±0.40) (Fig. 3C). In contrast, the ratio of VWF:RCo to ADAMTS13:AC significantly increased with the progression of liver diseases (0.9 ± 0.2 in healthy subjects; 0.7 ± 0.5 in chronic hepatitis; 1.6 ± 1.7 in Child A; 5.0 ± 5.7 in Child B; and 16.8 ± 28.2 in Child C) (Fig. 3D). On the other hand, platelet count decreased with the severity of chronic liver diseases: 15.9 ± 4.8 x10⁴/mm³ in chronic hepatitis; 9.6 ± 4.6 x10⁴/mm³ in Child A; 6.9 ± 2.4 x10⁴/mm³ in Child B; and 6.0 ± 2.3 x10⁴/mm³ in Child C.

Clinical and laboratory characteristics of 49 LC patients with a severe to mild reduction of plasma ADAMTS13:AC

Five patients (patients no. 1–5, Fig. 4A) were originally identified as being severely deficient in plasma ADAMTS13:AC by the VWF assay. Twenty-two patients (patient no. 6–27, Fig. 4B) showed a moderate deficiency (3–25% of the control), and the remaining 22 patients (no. 28–49, Fig. 4B) exhibited a mild reduction (25–50% of the control) of plasma ADAMTS13:AC by both the methods of VWF assay and the act-ELISA, without discordant results. Out of these 49 patients, the VWF assay (Fig. 4A, B) revealed that 26 (53.1%) had normal VWFs, 15 (30.6%) had degraded-VWFs, and the remaining eight (patients no. 3, 4, 11, 14, 16, 18, 23, and 26) (16.3%) had UL-VWFs. With respect to the comparison of clinical parameters according to VWF patterns, UL-VWFs-positive patients showed the lowest ADAMTS13:AC, and the highest values of serum creatinine, blood urea nitrogen, and blood ammonia (Table 3). In addition, LC patients with UL- and normal-VWFs showed higher levels of VWF:RCo and Child-Pugh score, and lower values of cholinesterase, total cholesterol, and hemoglobin than those with degraded-VWFs (Table 3).

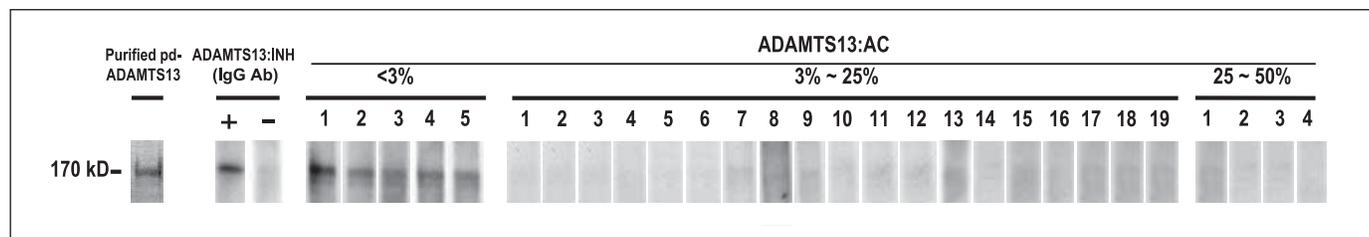


Figure 5: Detection of IgG-type autoantibodies specific to plasma derived-ADAMTS13 by Western blot. To detect IgG-type autoantibodies against ADAMTS13, we performed Western blot analysis using plasma derived (pd)-ADAMTS13 purified by A10-agarose immunoaffinity chromatography as described in *Materials and methods*. Heated plasma from a patient with acquired idiopathic TTP with IgG inhibitors against ADAMTS13 was used as a positive control, while that from a normal individual without ADAMTS13:INH was used as a negative con-

trol. Of the 28 LC patients with ADAMTS13:INH, five LC patients with severe ADAMTS13:AC deficiency (<3% of controls) exhibited in plasma a 170 kD-band, indicating the presence of IgG autoantibodies specific for purified pd-ADAMTS13 under non-reducing conditions. However, the IgG inhibitor against ADAMTS13 was not detected in the remaining 23 LC patients with moderate or mild deficiency of ADAMTS13:AC. pd=plasma derived, ADAMTS13:INH=ADAMTS13 inhibitor, IgG=immunoglobulin G, Ab=antibody, ADAMTS13:AC=ADAMTS13 activity.

ADAMTS13:INH and detection of IgG-type autoantibodies specific to pd-ADAMTS13 by WB

ADAMTS13:INH was detected in all five patients with severe ADAMTS13:AC deficiency (<3%), in 19 (86.4%) of 22 LC patients with moderate deficiency (3–25%), and in four (18.2%) of 22 LC patients with mild ADAMTS13:AC deficiency (25–50%). The inhibitory activity was 2.0 BU/ml (21) in a LC patient with TTP, and 3.0 BU/ml in a patient with severe ADAMTS13:AC deficiency, but in remaining patients the inhibitory activity showed marginal zone between 0.5 and 1.0 BU/ml. Of the 28 LC patients with ADAMTS13:INH, five LC patients with severe ADAMTS13:AC deficiency (<3%) exhibited in plasma a 170 kD-band, indicating the presence of IgG autoantibodies specific for purified pd-ADAMTS13 under non-reducing conditions (Fig. 5). However, the IgG inhibitor against ADAMTS13 was not detected in the remaining 23 LC patients with moderate or mild deficiency of ADAMTS13:AC (Fig. 5).

Clinical and laboratory characteristics of five LC patients with extremely low levels of plasma ADAMTS13:AC determined by the VWFM assay

All five patients with extremely low levels of plasma ADAMTS13:AC displayed signs of end stage liver disease (Table 4). Case 1 was associated with TTP (21) and case 2 had HRS and liver abscess. Case 3 was complicated by SBP and HRS, case 4 with direct invasion of HCC to transverse colon, and case 5 with SBP, HRS and portal thrombus. Cases 2, 4, and 5 had a marked inflammatory reaction, and Cases 2 and 4 showed a high concentration of IL-6. Plasma ADAMTS13:AC ranged from <0.5% to 15.9% as determined by act-ELISA. The plasma levels of ADAMTS13:AC determined by act-ELISA were in good accordance with those of plasma ADAMTS13:AG quantified both by the antigen-ELISA and by the Western blot analysis in cases 3, 4, and 5 (Table 4, Fig. 4C), but there was some discrepancy between the activity and antigen of ADAMTS13 in cases 1 and 2. UL-VWFM was detected in cases 3 and 4, whose VWF:RCo and the ratio of VWF:RCo to VWF:Ag was high (1.0, 0.8), whereas in the other three patients without UL-VWFM (cases 1, 2, and 5), VWF:RCo did not increase and the ratios were low (0.2–0.3). In contrast, cases 1 and 2 showed degraded

high-molecular-VWF multimers, which are present in normal plasmas. The ADAMTS13:INH levels in these five patients ranged from 0.5 to 3.0 BU/ml, and the inhibitor showed the IgG-type autoantibodies specific to pd-ADAMTS13 (Fig. 5).

Plasma cytokine levels

Plasma IL-6 concentrations were the highest in patients with Child C (51 ± 78 pg/ml) as compared with Child A (10 ± 5 pg/ml, $p < 0.05$), Child B (10 ± 6 pg/ml, $p < 0.05$), and chronic hepatitis (<7.8 pg/ml, $p < 0.05$), but IL-8 and TNF- α were below the detection limits in all patients. The levels of IL-6 in the 10 LC patients with SBP were among the highest (189 ± 403 pg/ml). In addition, there was a weak negative correlation between plasma levels of ADAMTS13:AC and IL-6 concentration in patients with chronic liver diseases ($r = -0.267$, $p < 0.002$) (Table 2).

Discussion

Until now, it has not been clear whether plasma ADAMTS13:AC would decrease in cirrhosis patients (17, 22, 23). In this study, we clearly demonstrated that plasma ADAMTS13:AC decreased with increasing cirrhosis severity in a large number of patients (Fig. 1B). We used two different techniques to measure ADAMTS13:AC, and values determined by the act-ELISA correlated well with those quantified by the VWFM assay (Fig. 1A). Additionally, plasma ADAMTS13:AC was closely correlated with plasma ADAMTS13:AG determined by the antigen-ELISA (Fig. 1C, D) as described in TTP patients (40, 41), confirming that both ADAMTS13 activity and antigen decreased with increasing cirrhosis severity. In contrast, Lisman et al. reported that both ADAMTS13 activity and antigen levels were highly variable; they did not distinguish between patients with varying degrees of cirrhosis (22). It is unclear why these studies reached differing conclusions, but one possible explanation relates to differences in disease etiology: a majority of our patients developed cirrhosis secondary to HCV infection, whereas in the Lisman et al. study, half of the patients suffered from alcohol abuse related cirrhosis. Alternatively, the techniques used to determine ADAMTS13:AC differed between our study (24, 28, 33) and

Table 4: Clinical characteristics in five cirrhotic patients with severe deficiency of ADAMTS13 determined by VWFm assay.

Pt.	Age/ Sex	Etiology	Child- Pugh Class/ Score	ADAMTS13:AC (%)		ADAMTS13:AG (%)		ADAMTS13: INH		VWF: Ag (%)	VWF: RCo (%)	VWF: RCo/ VWF:Ag	VWF: RCo/ ADAMT S13:AC	VWFm pattern
				VWFm assay	act- ELISA	ELISA	WB	act- ELISA (BU/ml)	WB (IgG)					
1	65/M	HCV	C/14	<3	6.4	13	58	2.0	+	155	25	0.2	3.9	degraded
2	64/M	Crypto- genic	C/10	<3	<0.5	28	35	3.0	+	332	75	0.2	>150	degraded
3	70/M	PBC	C/13	<3	6.3	10	10	1.0	+	277	280	1.0	44.4	unusually large
4	75/M	HCV	C/11	3	10.3	14	19	0.8	+	528	420	0.8	40.8	unusually large
5	70/M	HBV	C/14	3	15.9	17	13	0.5	+	305	84	0.3	5.3	normal
Pt.	Hb (g/dl)	WBC (/mm ³)	Plat. (10 ⁴ / mm ³)	CRP (mg/dl)	IL-6 (pg/ml)	IL-8 (pg/ml)	TNF α (pg/ml)	SBP	HRS	JIS score	Remarks			
1	5.1	7800	0.4	0.3	17	<15.6	<15.6	-	-	-	thrombotic thrombocytopenic purpura			
2	11.0	18600	8.3	23.4	455	18.2	<15.6	-	+	3	liver abscess, diabetes mellitus, hypertension			
3	9.6	3800	8.8	1.5	54	<15.6	<15.6	+	+	-				
4	9.2	8300	7.7	4.6	110	<15.6	<15.6	-	-	5	direct invasion of HCC to transverse colon			
5	7.1	10300	4.0	11.7	12	<15.6	<15.6	+	+	4	portal thrombus, diabetes mellitus			

HCV, hepatitis C virus; HBV, hepatitis B virus; PBC, primary biliary cirrhosis; ADAMTS13:AC, ADAMTS13 activity; ADAMTS13:AG, ADAMTS13 antigen; ADAMTS13:INH, ADAMTS13 inhibitor; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity; VWFm, von Willebrand factor multimer; ELISA, enzyme-linked immunosorbent assay; WB, Western blot; BU, Bethesda Units; Hb, hemoglobin; WBC, white blood cell count; Plat, platelet count; CRP, C-reactive protein; IL-6, interleukin-6; IL-8, interleukin-8; TNF- α , tumor necrosis factor- α ; SBP, spontaneous bacterial peritonitis; HRS, hepatorenal syndrome; JIS, The Japan Integrated Staging score obtained via the summation of Child-Pugh score and tumor stage score (32); HCC, hepatocellular carcinoma.

theirs, which might explain the high variability they observed. They used the collagen binding assay, which can be influenced by the increase in plasma VWF:Ag usually found in LC patients (23), whereas our act-ELISA is neither influenced by a large amount of VWF:AG nor by hyperbilirubinemia (28). Our present data indicating that ADAMTS13:AC decreases with increasing cirrhosis severity are consistent with those recently reported by Feys et al. (23) who analyzed ADAMTS13:AC using modified collagen binding methods.

Additionally, in our study plasma ADAMTS13:AC was remarkably low in LC patients with hepatic encephalopathy, hepatorenal syndrome and refractory ascites. Furthermore, the decreased activity was also seen in patients with higher JIS scores (4 to 5), indicating that ADAMTS13:AC was markedly reduced when cirrhosis was complicated by diffuse HCC (Fig. 2). It was recently reported that disseminated malignancies are associated with a moderately decreased ADAMTS13:AC (15% of the control), implying that a mechanism regulating the primary platelet-tumor adhesive interactions is involved in the metastatic process (42). In addition, using univariate analysis, we showed that ADAMTS13:AC significantly correlated with 17 clinical variables (Table 2). Among these factors excluding Child-Pugh score, multivariate analysis identified spleen volume, blood ammonia and serum creatinine as the independent factors contributing to the changes in ADAMTS13:AC. When Child-Pugh score was incorporated into the analysis instead of the three parameters used to generate the score, the Child-Pugh score and spleen

volume were independently selected, indicating that ADAMTS13:AC is closely related to the severity of liver disease and splenomegaly in cirrhotic patients.

Alternatively, the plasma levels of VWF:Ag were increased by 2.5-fold in patients with chronic hepatitis, and 3- to 5-fold in both early and advanced LC (Fig. 3A), as previously reported (9, 10). The marked elevation of plasma VWF:Ag in LC patients may be partly attributable to increased endothelial production induced by endotoxin (9, 43) and/or increased synthesis by extrahepatic endothelial cells (44). The VWF:RCo was higher (Fig. 3B), but the VWF:RCo relative to VWF:Ag was lower in LC patients (Fig. 3C) than in healthy subjects, indicating that increased VWF:Ag appears less functional in cirrhosis patients. These findings are consistent with previous reports (22). Nevertheless, it is interesting to note that the VWF:RCo relative to ADAMTS13:AC progressively increased with worsening chronic liver diseases (Fig. 3D), suggesting an enhanced thrombogenesis as liver dysfunction and thrombocytopenia progresses (Fig. 3E). We found that decreased platelet counts were coincident with decreased plasma ADAMTS13:AC (Table 2), which suggests an additional mechanism for thrombocytopenia related to hypercoagulability in patients with advanced cirrhosis, distinct from hypersplenism (11) and decreased production of thrombopoietin (12, 13).

There were three different VWFm patterns in 49 LC patients with lower ADAMTS13:AC (<50 % of controls): normal-VWFm was detected in 53%, degraded-VWFm in 31%, and

UL-VWFM in 16% (Fig. 4). UL-VWFM-positive patients showed the lowest ADAMTS13:AC, and the highest levels of serum creatinine, blood urea nitrogen, and blood ammonia (Table 3). Furthermore, LC patients with UL- and normal-VWFM showed higher levels of VWF:RCo, lower functional liver capacity, and a higher prevalence of anemia relative to those with degraded-VWFM (Table 3). From these results, plasma VWFM appears to shift from degraded- to normal-VWFM, and finally to the UL-VWFM as functional liver capacity and renal function deteriorates. ADAMTS13 may be gradually consumed and decreased, resulting in increased VWF:Ag concomitant with decreased ADAMTS13:AC, leading to an appearance of UL-VWFM in advanced cirrhosis. Taken together, advanced cirrhosis may be a predisposing state for platelet microthrombi formation, even in the absence of clinically overt thrombotic events. In fact, portal or hepatic vein thrombosis is often observed in advanced LC patients routinely screened with Doppler ultrasound (14), as well as in cirrhotic liver tissue removed at transplantation (15). Moreover, microthrombi were found in one or more organs in half of cirrhotic livers at autopsy (16), which is consistent with our hypothesis.

Remarkably, using the VWFM assay we found severe deficiency of ADAMTS13:AC (<3% of controls) corresponding to the level of TTP in five LC patients with end-stage liver disease. One of them showed apparent TTP (21), and others were complicated by HRS, SBP, a marked inflammation together with cytokinemia, and advanced HCC (Table 4). In general, various clinical conditions, including infection, malignancies, and certain drugs, can lead to acquired TTP (45). In advanced cirrhosis, endotoxemia is frequently detected (9), and SBP sometimes occurs (31). HCC is highly complicated as the cirrhotic stage progresses (46), indicating a high risk state for platelet microthrombi formation. Furthermore, in cases 3, 4 and 5, the ADAMTS13:AC by the act-ELISA were in good accordance with ADAMTS13:AG (Table 4, Fig. 4C), but in cases 1 and 2, there was some discrepancy between them, indicating that ADAMTS13:AG was considerably present in these cases, even if the activity was extremely low.

Plasma ADAMTS13:AC may decrease in advanced cirrhosis due to decreased production of ADAMTS13 in HSCs (47), enhanced consumption to degrade large quantities of VWF:Ag, and/or its plasma inhibitor (5, 6), the binding site of which is considered to be cysteine-rich/spacer domains (48). We observed plasma ADAMTS13:INH in 83% of patients with severe to moderate ADAMTS13:AC deficiency, but its inhibitory activity showed marginal zone between 0.5 and 1.0 BU/ml in most cases except a TTP patient (2.0 BU/ml) (Table 4, case 1) (21) and a patient with severe ADAMTS13:AC deficiency (3.0 BU/ml) (Table 4, case 2). Remarkably, we could detect the IgG-type autoantibodies specific to purified pd-ADAMTS13 by Western blot in five end-stage LC patients with severe ADAMTS13:AC deficiency (<3%) (Fig. 5), as described in TTP (5, 6, 49), but not in those with moderate or mild deficiency of the protease (Fig. 5). One of them (case 1, Table 4) certainly had characteristic clinical features of TTP with the IgG inhibitor against ADAMTS13 (0.4 BU/mg IgG using purified IgG from the patient's plasma) as previously reported (21). The remaining four

patients did not show any apparent clinical features of TTP, but seem to be indistinguishable from the typical TTP patient (Case 1, Table 4) from the points of ADAMTS13:AC and its inhibitor. These results indicate that some end-stage LC patients who have extremely low ADAMTS13:AC with the IgG inhibitor against ADAMTS13 might be under the condition similar to TTP, or might reflect "subclinical" TTP. With respect to the autoantibodies in patients with HCV-associated liver diseases, there is a general consensus that the overall prevalence of serum non-organ-specific autoantibodies are significantly higher in patients with HCV (about one third of all cases) than in both healthy subjects and patients with HBV (50–52). The etiology of our five end-stage LC patients with IgG-type autoantibodies was HCV in two, HBV in one, PBC in one, and cryptogenic in one, indicating that the presence of the autoantibodies against ADAMTS13 might be more associated with the disease progression. The decrease in ADAMTS13:AC would mainly be attributable to a decreased synthesis of ADAMTS13 due to liver failure and/or enhanced consumption to degrade large quantities of VWF:Ag, but further studies are warranted in order to clarify which kind of inhibitor other than the IgG inhibitor would be involved in patients with lower ADAMTS13:AC.

In addition, ADAMTS13:AC negatively correlated with plasma IL-6 concentrations, which is thought to promote the decrease in the ADAMTS13:AC (53) *in vitro*. The inflammation caused by SBP, endotoxemia, or other processes may thus be an important factor precipitating the decreased plasma ADAMTS13:AC in advanced cirrhosis (54). A recent study suggested that in patients with sepsis-induced disseminated intravascular coagulation, decreased ADAMTS13:AC could act together with UL-VWFM to contribute to the development of renal failure (55). In our patients with hepatorenal syndrome frequently complicated with SBP, the ADAMTS13:AC was extremely low, suggesting that markedly decreased ADAMTS13:AC could be a precipitating factor for the development of hepatorenal syndrome.

In summary, both plasma ADAMTS13 activity and antigen levels decreased with increasing severity of liver cirrhosis. An imbalance between the decreased ADAMTS13:AC and its increased substrate may reflect the predisposing state for platelet thrombi formation in patients with advanced liver cirrhosis.

Abbreviations

ADAMTS, a disintegrin-like and metalloproteinase domain with thrombospondin type-1 motif; ADAMTS13:AC, ADAMTS13 activity; ADAMTS13:AG, ADAMTS13 antigen; ADAMTS13:INH, ADAMTS13 inhibitors; ELISA, enzyme-linked immunosorbent assay; HCC, hepatocellular carcinoma; IL-6, interleukin 6; IL-8, interleukin 8; JIS, Japan Integrated Staging; SBP, spontaneous bacterial peritonitis; TNF- α , tumor necrosis factor- α ; TTP, thrombotic thrombocytopenic purpura; UL-VWFM, unusually large von Willebrand factor multimer; VWF, von Willebrand factor; VWF:AG, von Willebrand factor antigen; VWFM, VWF multimer; VWF:RCo, VWF ristocetin cofactor activity.

References

1. Levy GG, et al. Mutations in a member of the ADAMTS13 gene family cause thrombotic thrombocytopenic purpura. *Nature* 2001; 413: 488–494.
2. Zheng X, et al. Structure of von Willebrand factor-cleaving protease (ADAMTS 13), metalloproteinase involved in thrombotic thrombocytopenic purpura. *J Biol Chem* 2001; 276: 41089–41163.
3. Moake JL. Thrombotic microangiopathies. *N Engl J Med* 2002; 347: 589–599.
4. Kokame K, et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci USA* 2002; 99: 11902–11907.
5. Furlan M, et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 1998; 339: 1578–1584.
6. Tsai H-M, et al. Antibody to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 1998; 339: 1585–1594.
7. Moschowitz E. Hyaline thrombosis of the terminal arterioles and capillaries: a hitherto undescribed disease. *Proc NY Pathol Sac* 1924; 24: 21–24.
8. Uemura M, et al. Localization of ADAMTS13 to the stellate cells of human liver. *Blood* 2005; 106: 922–924.
9. Ferro D, et al. High plasma levels of von Willebrand factor as a marker of endothelial perturbation in cirrhosis: relationship to endotoxemia. *Hepatology* 1996; 23: 1377–1383.
10. Albornoz L, et al. Von Willebrand factor could be an index of endothelial dysfunction in patients with cirrhosis: relationship to degree of liver failure and nitric oxide levels. *J Hepatol* 1999; 30: 451–455.
11. Aster RH. Pooling of platelets in the spleen: role in the pathogenesis of “hypersplenic” thrombocytopenia. *J Clin Invest* 1966; 45: 645–657.
12. Peck-Radosavljevic M, et al. Thrombopoietin induces rapid resolution of thrombocytopenia after orthotopic liver transplantation through increased platelet production. *Blood* 2000; 95: 795–801.
13. Schoffski P, et al. Thrombopoietin serum levels are elevated in patients hepatitis B/C infection compared to other causes of chronic liver diseases. *Liver* 2002; 22: 114–120.
14. Amitrano L, et al. Risk factors and clinical presentation of portal vein thrombosis in patients with liver cirrhosis. *J Hepatol* 2004; 40: 736–741.
15. Wanless IR, et al. Hepatic and portal vein thrombosis in cirrhosis: possible role in development of parenchymal extinction and portal hypertension. *Hepatology* 1995; 21: 1238–1247.
16. Oka K, et al. Intravascular coagulation in autopsy cases with liver diseases. *Thromb Haemost* 1979; 42: 564–570.
17. Mannucci PM, et al. Changes in health and disease of the metalloproteinase that cleaves von Willebrand factor. *Blood* 2001; 98: 2730–2735.
18. Park YD, et al. Impaired activity of plasma von Willebrand factor-cleaving protease may predict the occurrence of hepatic veno-occlusive disease after stem cell transplantation. *Bone Marrow Transplant* 2002; 29: 789–794.
19. Uemura M, et al. Decreased Activity of plasma ADAMTS13 may contribute to the development of liver disturbance and multiorgan failure in patients with alcoholic hepatitis. *Alcohol Clin Exp Res* 2005; 29: 264S–271S.
20. Ko S, et al. Plasma ADAMTS13 activity may predict early adverse events in living donor liver transplantation: observations in 3 cases. *Liver Transpl* 2006; 12: 859–869.
21. Yagita M, et al. Development of ADAMTS-13 inhibitor in a patient with hepatitis C virus-related liver cirrhosis causes thrombotic thrombocytopenic purpura. *J Hepatol* 2005; 42: 420–421.
22. Lisman T, et al. Elevated levels of von Willebrand factor in cirrhosis support platelet adhesion despite reduced functional capacity. *Hepatology* 2006; 44: 53–61.
23. Feys HB, et al. ADAMTS13 activity to antigen ratio in physiological and pathological conditions associated with an increased risk of thrombosis. *Br J Haematol* 2007; 138: 534–540.
24. Furlan M, et al. Deficiency activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. *Blood* 1997; 89: 3097–3103.
25. Studt JD, et al. Fatal congenital thrombotic thrombocytopenic purpura with apparent ADAMTS13 inhibitor: in vitro inhibition of ADAMTS13 activity by hemoglobin. *Blood* 2005; 105: 542–544.
26. Kokame K, et al. FRETTS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol* 2005; 129: 93–100.
27. Eckmann CM, et al. Bilirubin oxidase as a solution for the interference of hyperbilirubinemia with ADAMTS-13 activity measurement by FRETTS-VWF73 assay. *J Thromb Haemost* 2007; 5: 1330–1331.
28. Kato S, et al. Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity. *Transfusion* 2006; 46: 1444–1452.
29. Pugh RNH, et al. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; 60: 646–649.
30. Henderson JM, et al. Measurement of liver and spleen volume by computed tomography. *Radiology* 1981; 141: 525–527.
31. Arroyo V, et al. Definition and diagnostic criteria of refractory ascites and hepatorenal syndrome in cirrhosis. *International ascites club. Hepatology* 1996; 23: 164–176.
32. Kudo M, et al. Validation of a new prognostic staging system for hepatocellular carcinoma: the JIS score compared with the CLIP score. *Hepatology* 2004; 40: 1396–1405.
33. Mori Y, et al. Predicting response to plasma exchange in patients with thrombotic thrombocytopenic purpura with measurement of vWF-cleaving protease activity. *Transfusion* 2002; 42: 572–580.
34. Yagi H, et al. Plasma Levels of ADAMTS13 antigen determined with an enzyme immunoassay using a neutralizing monoclonal antibody parallel ADAMTS13 activity levels. *Int J Hematol* 2007; 85: 403–407.
35. Ishizashi H, et al. Quantitative Western blot analysis of plasma ADAMTS13 antigen in patients with Upshaw-Schulman syndrome. *Thromb Res* 2007; 120: 381–386.
36. Macfarlane DE, et al. A method for assaying von Willebrand factor (ristocetin cofactor). *Thromb Diath Haemorrh* 1975; 34: 306–308.
37. Warren CM, et al. Vertical agarose gel electrophoresis and electroblotting of high-molecular-weight proteins. *Electrophoresis* 2003; 24: 1695–1702.
38. Kasper CK, et al. A more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 1975; 34: 869–872.
39. Hiura H, et al. Immuno-purification of plasma ADAMTS13 and its physico-chemical characterization. *International Society on Thrombosis and Haemostasis Congress, Geneva, 2007. P-M-295.*
40. Liu F, et al. Alteration of ADAMTS13 antigen levels in patients with idiopathic thrombotic thrombocytopenic purpura, idiopathic thrombocytopenic purpura and systemic lupus erythematosus. *Thromb Haemost* 2006; 95: 749–750.
41. Rieger M, et al. Relation between ADAMTS13 activity and ADAMTS13 antigen levels in healthy donors and patients with thrombotic microangiopathies (TMA). *Thromb Haemost* 2006; 95: 212–220.
42. Oleksowicz L, et al. Deficiency of von Willebrand's factor-cleaving protease in patients with disseminated malignancies. *Cancer Res* 1999; 59: 2244–2250.
43. Schorer AE, et al. Interleukin 1 or endotoxin increases the release of von Willebrand factor from human endothelial cells. *Brit J Haematol* 1987; 67: 193–197.
44. Tornai I, et al. Endothelium releases more von Willebrand factor and tissue-type plasminogen activator upon venous occlusion in patients with liver cirrhosis than in normals. *Haemostasis* 1993; 23: 58–64.
45. Matsumoto M, et al. The Japanese experience with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *Semin Hematol* 2004; 41: 68–74.
46. Ikeda K, et al. A multivariate analysis of risk factors for hepatocellular carcinogenesis: A prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* 1993; 18: 47–53.
47. Kume Y, et al. Hepatic stellate cell damage may lead to decreased plasma ADAMTS13 activity in rats. *FEBS Letters* 2007; 581: 1631–1634.
48. Luken BM, et al. The spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with thrombotic thrombocytopenic purpura. *Thromb Haemost* 2005; 93: 267–274.
49. Tsai HM, et al. ADAMTS13-binding IgG are present in patients with thrombotic thrombocytopenic purpura. *Thromb Haemost* 2006; 95: 886–892.
50. Lenzi M, et al. Prevalence of non-organ-specific autoantibodies and chronic liver disease in the general population: a nested case-control study of the Dionysos cohort. *Gut* 1999; 45: 328–329.
51. Hsieh MY, et al. Antinuclear antibody is associated with a more advanced fibrosis and lower RNA levels of hepatitis C virus in patients with chronic hepatitis C. *J Clin Pathol* 2008; 61: 333–337.
52. Clifford BD, et al. High prevalence of serological markers of autoimmunity in patients with chronic hepatitis C. *Hepatology* 1995; 21: 613–619.
53. Bernardo A, et al. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. *Blood* 2004; 104: 100–106.
54. Reiter RA, et al. Changes in ADAMTS13 (von-Willebrand-factor-cleaving protease) activity after induced release of von Willebrand factor during acute systemic inflammation. *Thromb Haemost* 2005; 93: 554–558.
55. Ono T, et al. Severe secondary deficiency of von Willebrand factor-cleaving protease (ADAMTS13) in patients with sepsis-induced disseminated intravascular coagulation: its correlation with development of renal failure. *Blood* 2006; 107: 528–534.